Clinical aspects of cell death in breast cancer: the polyamine pathway as a new target for treatment

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Abstract
Because intracellular polyamines have a critical role in cell proliferation and death pathways, the polyamine metabolic pathway represents a potential target for intervention in cancers. A number of polyamine analogues have been identified that downregulate polyamine synthesis and enhance polyamine catabolism, thereby depleting intracellular polyamines. Treatment of human breast cancer cell lines in culture with these analogues has been shown to decrease cell proliferation and induce programmed cell death. Phase I studies with one analogue are now complete, setting the stage for phase II trials to determine efficacy, in addition to preclinical studies to examine combinations of polyamine analogues and conventional cytotoxics.

Introduction
Metastatic breast cancer is a common disease. A major clinical problem is that tumours that are initially responsive to both hormonal and chemotherapeutic approaches generally progress to more aggressive forms that are poorly responsive to either category of agents. The need for anti-neoplastic agents with novel mechanisms of action is therefore great.

Intracellular polyamines have an important role in the proliferation of normal and malignant cells. The recognition of their critical role in cell growth and differentiation has led to the development of several inhibitors of polyamine biosynthesis, as exemplified by difluoromethylornithine (DFMO), which is directed against ornithine decarboxylase (ODC), the first enzyme in the polyamine biosynthetic pathway (Mamont et al. 1978, Danzin et al. 1982, Casero et al. 1984, Porter & Sufrin 1986, Marton & Pegg 1995). Both the chemotherapeutic and chemopreventive effects of DFMO have been the focus of several clinical trials.

Recently, however, attention has been focused on other steps in the polyamine metabolic pathway as potential targets for intervention. In particular, the \( N,N' \)-bis(ethyl) analogues of spermine have been found to downregulate ODC, deplete intracellular polyamine pools, and inhibit cell growth (Porter et al. 1987). Depletion of polyamine pools and subsequent growth inhibition appear to be mediated in part through the induction of the enzyme, spermidine/spermine \( N^1 \)-acyltransferase (SSAT), the rate-limiting enzyme in the polyamine catabolic pathway (Porter et al. 1991, Casero & Pegg 1993). Although they readily accumulate in cells, a key feature of these analogues is that they do not substitute for the depleted natural polyamines (Porter et al. 1991). \( N^1 \)-\( N^{12} \)-bis-(ethyl)spermine (BESpm) is a compound representative of this family of agents. In addition, several asymmetrically alkylated analogues that are structurally similar to the bis(ethyl)polyamines have been synthesised as potential anti-tumour agents (Saab et al. 1993). Structures for some of the substituted and unsubstituted polyamine analogues are shown in Fig. 1.

The importance of polyamine biosynthesis and action in breast cancer has been extensively studied and reviewed (Manni 1994). Because of these data, in addition to preclinical data suggesting anti-neoplastic effects of these symmetric and asymmetric polyamine analogues in a
variety of tumour types, including non-small cell lung cancer (Casero et al. 1989), melanoma (Bernacki et al. 1991), pancreatic cancer (Chang et al. 1991), and ovarian cancer (Bernacki et al. 1995), the effects of these compounds on the growth of human breast cancer cell lines in tissue culture have been assessed.

**Growth effects of polyamine analogues**

The prototype analogue, BESpm, significantly inhibits the growth of six human breast cancer cell lines (MCF-7, T47D, ZR-75-1, MDA-MB-231, MDA-MB-468, and Hs578t) with 50% inhibitory concentrations in the low micromolar range (Davidson et al. 1993). The degree of inhibition does not correlate with hormone receptor status. Detailed studies with the oestrogen receptor (ER)-positive MCF-7 and ER-negative Hs578t cell lines showed similar dose-response curves, with concentrations of 1-10 µM resulting in maximal growth inhibition. Growth inhibition of both cell lines was associated with an 8- to 12-fold induction of the polyamine catabolic enzyme, SSAT, and progressive decrease in polyamine concentrations over 6 days, although steady-state concentrations of BESpm were achieved within 24 h. Similar studies on WTMCF-7 cells and the doxorubicin-resistant, ER-negative AdrMCF-7 cells derived from WTMCF-7 by step-wise incubation in doxorubicin show that acquisition of resistance to hormonal or doxorubicin treatment was not associated with resistance to the growth-inhibitory effects of BESpm. Indeed, the extent of growth inhibition, SSAT induction, and polyamine depletion after BESpm treatment was similar between the two cell lines. Thus, in aggregate, these initial results suggested that BESpm exerts similar growth-inhibitory effects against both

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**Figure 1** Structures of spermine and some polyamine analogues. BESpm, $N^{1},N^{12}$-bis(ethyl)spermine; BENSpm, $N^{1},N^{11}$-bis(ethyl)norspermine; CPENSpm, $N^{1}$-ethyl-$N^{11}$-((cyclopropyl)methyl)-4,8-diazaundecane; CHENSpm, $N^{1}$-ethyl-$N^{11}$-[(cycloheptyl)methyl]-4,8-diazaundecane.

**Figure 2** Dose-response curves for BENSpm treatment of human breast cancer cells. MDA-MB-468 (A) and MCF-7 cells (B) in DMEM and 5% FCS were grown in the presence or absence of BENSpm for 120 h. Cells were harvested and counted by Coulter counter; results are expressed as percentage of untreated control cell number. Means±S.E. for triplicate determinations from a representative experiment are shown.
hormone-responsive and -unresponsive human breast cancer cells. In addition, resistance to one chemotherapeutic agent, doxorubicin, was not associated with resistance of the polyamine analogue (Davidson et al. 1993).

The activity of the related compound, \( N^{1}, N^{11}\)-bis(ethyl)norspermine or BENSpm (also known as \( N^{1}, N^{11}\)-diethylnorspermine or DENSpm), has also been studied in selected human breast cancer cell lines. As shown in Fig. 2, treatment of MDA-MB-468 and MCF-7 cells resulted in growth inhibition, with an IC\(_{50}\) of 1-10 \( \mu \)M after 120 h of chronic exposure. Treatment of MDA-MB-468 cells was associated with depletion of natural polyamines, intracellular accumulation of BENSpm, and a 400-fold induction of the polyamine catabolic enzyme, SSA T (Table 1). In addition, in preliminary studies, short-term explants of primary human breast cancers have been exposed to 10 \( \mu \)M BENSpm overnight in tissue culture and evidence of induction of the SSA T enzyme has been observed by immuno-histochemistry (data not shown). Thus, findings from established human breast cancer cell lines in culture appear also to hold true in short-term cultures of malignant breast tissue. The agent, BENSpm, is of particular interest, as it has been used in several phase I trials in humans, and a dose and schedule for phase II testing have been selected, as discussed below.

### Polyamine analogues as mediators of programmed cell death

A relationship between polyamines and the process of programmed cell death has been suggested by several experimental findings. These include data suggesting that: (1) spermidine and spermine are able to stabilise chromatin (Marton & Morris 1987, Porter & Janne 1987), (2) polyamine-depleted cells undergo changes in chromatin and DNA structure (Marton & Morris 1987, Porter & Janne 1987), and (3) spermine can protect against programmed cell death in thymocytes (Brüne et al. 1991).

As a consequence, the possibility that polyamine analogues might induce programmed cell death in addition to their effects on proliferation was investigated, using an asymmetric polyamine analogue, \( N^{1}\)-ethyl-\( N^{11}\)-(cyclopropyl)methyl)-4,8-diazaundecane (CPENSpm).

McCloskey et al. (1995) assessed the sensitivity to continuous CPENSpm exposure of the same six breast cancer cell lines evaluated in the BESpm study above. All cell lines exhibited concentration-dependent growth inhibition with IC\(_{50}\) values of 0.2-1.3 \( \mu \)M. Again, there was no relationship between oestrogen receptor status and sensitivity to CPENSpm. Given the significant growth inhibitory activity of CPENSpm, the possibility that part of this effect was a result of induction of programmed cell death was investigated. Fragmentation of genomic DNA to high molecular weight fragments (\( \geq \)50 kb) is characteristic of programmed cell death and may represent the committed step of the pathway. Field inversion gel electrophoresis was used to assess whether prolonged (4-day) exposure to CPENSpm could induce such DNA cleavage. Concentration-dependent high molecular weight DNA fragmentation was detected in all six cell lines after chronic exposure to CPENSpm and was not seen in untreated control cells. Because fragmentation of DNA into oligonucleosomal pieces is also associated with programmed cell death in many cell systems, DNA isolated from CPENSpm-treated MCF-7 and MDA-MB-468 cells was also examined for evidence of such a change. Oligonucleosomal DNA fragmentation was detected in both cell lines after exposure to 10 \( \mu \)M CPENSpm for 96 h, but was not observed in untreated samples.

### Table 1 Effects of BENSpm on polyamine pools and SSA T activity in MDA-MB-468 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Polyamines (nmol/mg protein)</th>
<th>SSAT activity (pmol/mg protein/min)</th>
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<tbody>
<tr>
<td></td>
<td>Putrescine</td>
<td>Spermidine</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>Control</td>
<td>1.95</td>
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<tr>
<td></td>
<td>BENSpm</td>
<td>0.96</td>
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<tr>
<td>Experiment 2</td>
<td>Control</td>
<td>2.15</td>
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<td></td>
<td>BENSpm</td>
<td>1.6</td>
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After incubation of exponentially growing MDA-MB-468 cells in the presence or absence of 10\( \mu \)M BENSpm for 24 h, intracellular polyamine concentrations and SSA T activity were determined as described previously (Davidson et al. 1993, McCloskey et al. 1995). Values are averages of two determinations from two independent experiments. ND, not determined.
control mechanisms of action for these compounds exist. Detailed studies using the MDA-MB-468 cell line confirmed that polyanamine depletion and induction of SSAT were features of CPENSpm-induced programmed cell death in this cell line. Interestingly, SSAT activity was not significantly induced in any of the other five breast cancer cell lines tested, suggesting that CPENSpm cytotoxicity is not solely dependent on SSAT induction as a means of depleting polyamine.

Cytotoxic effects of CPENSpm are not limited to breast cancer cell lines. Indeed, treatment of the NCI-H157 human non-small-cell lung carcinoma cell line with either BENSpm or CPENSpm resulted in polyamine depletion, SSAT induction, and morphological and biochemical changes consistent with activation of programmed cell death pathways (McCloskey et al. 1996). Studies using this cell line have also demonstrated that catalysis of polyamines by the SSAT/polyamine oxidase pathway has H2O2 as one product (Ha et al. 1997). Furthermore, the findings of studies utilizing inhibitors of this pathway suggested that programmed cell death induced by CPENSpm may be due, in part, to oxidative stress as a result of H2O2 production (Ha et al. 1997). This observation is not characteristic of programmed cell death pathways induced by all polyamine analogues in this cell line, in that treatment with another asymmetrically substituted analogue, N1-ethyl-N11,11-(cycloheptyl)-methyl]-4,8,12-diazaundecane (CHENSpm), also led to high molecular weight DNA fragmentation, but through a mechanism that does not appear to involve oxidative stress; rather, CHENSpm-treated, but not CPENSpm-treated, H157 cells showed evidence of cell cycle changes - specifically, induction of a G2/M block within 16 h of treatment (Ha et al. 1997). Thus it is likely that multiple mechanisms of action for these compounds exist.

**Clinical application of polyamine analogues in breast cancer**

Phase I testing of one polyamine analogue has now been completed. BENSpm, also known as DENSpm, has been studied in three different phase I studies, encompassing once-daily, twice-daily, and thrice-daily doses for 5 days of every 21-28 day cycle (Creaven et al. 1997, Ettinger et al. 1998, unpublished observations). Dose-limiting toxicities were predominantly those related to gastrointestinal or neurological toxicity. Haematological toxicity was not observed. These studies have led to identification of a dose of 100 mg/m2 given as an untravenous bolus a single daily for 5 days of every 28 day cycle for use in phase II studies. Pharmacokinetic studies using this dose and schedule have shown that the maximal plasma concentration of BENSpm achieved is 21-25 µM, a concentration that has been shown to have biological activity in preclinical models as summarised above. Thus phase II studies examining the ability of BENSpm to induce tumour regression or maintain a progression-free state can begin shortly.

Given the lack of haematological toxicity observed with BENSpm in initial human studies, a second area for study is the possibility for combined therapy using polyamine analogues with more conventional cytotoxics already used for breast cancer treatment. Current studies are evaluating the effects in breast cancer cell lines in culture of combinations or sequences of polyamine analogues and agents such as doxorubicin, 5-fluorouracil, and the taxanes, looking for evidence of synergistic actions. It will then be possible to test promising combinations in animal model systems, in preparation for human trials.

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